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Patent- og

Use of Acid-stable Proteases in Animal Feed.

Technical Field

The present invention relates to the use of acid-stable, serine proteases in animal feed, and to the use of such proteases for the solubilisation and/or degradation of vegetable proteins.

Proteins are essential nutritional factors for animals and humans. Most livestock and many human beings get the necessary proteins from vegetable protein sources. Important vegetable protein sources are e.g. oilseed crops, legumes and cereals.

When e.g. soybean meal is included in the feed of mono-gastric animals such as pigs and poultry, a significant proportion of the soybean meal solids is not digested. E.g., apparent ileal protein digestibilities of only 77% and 84% have been reported in piglets and growing pigs, respectively.

The stomach of mono-gastric animals and many fish exhibits a strongly acidic pH. Most of the protein digestion, however, occurs in the small intestine. A need therefore exists for an acid-stable protease that can survive passage of the stomach.

Background Art

The use of proteases in animal feed, or to treat vegetable proteins, is known from the following documents:

WO95/28850 discloses i.a. an animal feed additive comprising a phytase and a proteolytic enzyme. Various proteolytic enzymes are specified at p. 7.

WO96/05739 discloses an enzyme feed additive comprising xylanase and a protease. Suitable proteases are listed at p. 25.

WO95/02044 discloses i.a. proteases derived from Aspergillus aculeatus, as well as the use in animal feed thereof.

US 3,966,971 discloses a process of obtaining protein from a vegetable protein source by treatment with an acid phytase and optionally a proteolytic enzyme. Suitable proteases are specified in column 2.

These proteases, however, are not acid-stable and/or not serine proteases.

Brief Description of the Invention

Several proteases have now been identified which are found to be very acid-stable, and expectedly of an improved performance in animal feed. These proteases belong to a group of proteases known as serine proteases.

Brief Description of Drawings

The present invention is further illustrated by reference to the accompanying drawings, in which:

Fig. 1 shows pH-stability curves, viz. residual protease activity of five proteases (two acid-stable, serine proteases, and three reference proteases) after incubation for 2 hours, at a temperature of 37°C, and at pH-values in the range of pH 2 to pH 11; the activity is relative to residual activity at pH 9.0, and 5°C;

Fig. 2 shows pH-activity curves, viz. protease activity between pH 3 and pH 11, relative to the protease activity at pH-optimum, of the same five proteases; and

Fig. 3 shows temperature-activity curves, viz. protease activity between 15°C and 80°C, relative to protease activity at the optimum temperature, of the same five proteases.

Detailed description of the invention

The term ''protease'' as used herein is an enzyme that hydrolyses peptide bonds. Proteases are also called e.g. ''peptidases'', ''proteinases'', ''peptide hydrolases,'' or ''proteolytic enzymes.''

Preferred proteases for use according to the invention are of the endo-type that act internally in polypeptide chains (endopeptidases).

Included in the above definition of protease are any enzymes belonging to the EC 3.4 enzyme group (including each of the thirteen sub-subclasses thereof) of the EC list (Enzyme Nomenclature 1992 from NC-IUBMB, 1992), as regularly supplemented and updated, see e.g. the World Wide Web (WWW) at http://www.chem.qmw.ac.uk/iubmb/enzyme/index.html.

Proteases are classified on the basis of their catalytic mechanism into the following groupings: serine proteases (S), cysteine proteases (C), aspartic proteases (A), metalloproteases (M), and unknown, or as yet unclassified, proteases (U), see Handbook of Proteolytic Enzymes, A.J.Barrett, N.D.Rawlings, J.F.Woessner (eds), Academic Press (1998), in particular the general introduction part.

The term ''serine protease'' as used herein refers to ''serine peptidases and their clans'' as defined in the above Handbook. In the 1998 version of this handbook, serine peptidases and their clans are dealt with in chapters 1-175.

The following are particular embodiments of the serine proteases for use according to the invention:

Peptidases in which the catalytic mechanism depends upon the hydroxyl group of a serine residue acting as the nucleophile that attacks the peptide bond;

threonine dependent peptidases

peptidases of clan SA (Chapters 2 and 76 of the above Handbook); clan SB (Chapter 93); clan SC (Chapter 124); clan SE (Chapter 141); clan SF (Chapter 151); clan SH (Chapter 161); clan TA (Chapter 167); or not yet assigned to a clan (Chapter 175).

Thus, the few known families of threonine-dependent peptidases), as well as proteases not yet assigned to a clan, are included in the term of ''serine proteases.''

The following protease families are further particular embodiments of the serine proteases for use according to the invention (cf. the above Handbook): S1, S2, S3, S6, S7, S29, S30, S31, S32, S8, S9, S10, S15, S28, S33, S37, S11, S12, S13, S24, S26, S41, S44, S21, T1, S42, S14, S16, S18, S19, S34, S38, S39, S43 - or any sub-combination thereof.

Family S8 of clan SB are the so-called subtilisins.

Protease ''activity'' can be measured using any assay, in which a substrate is employed, that includes peptide bonds relevant for the specificity of the protease in question. Assay-pH and assay temperature are likewise to be adapted to the protease in question. Examples of assay-pH-values are pH 5, 6, 7, 8, 9 or 10. Examples of assay temperatures are 30, 35, 37, 40, 45 or 50°C.

Examples of protease substrates are casein, and pNA-substrates, such as Suc-AAPF-pNA (available e.g. from Bachem L-1400), or Boc-VLGR-pNA (Bachem L-1205), or Suc-AAPE-pNA (Bachem L-1710). Another example is Protazyme AK (Megazyme T-PRAK). The capital letters in the pNA-substrates refer to the one-letter amino acid code. For pH-activity and pH-stability studies, the pNA-substrates are preferred, whereas for temperature-activity studies, the Protazyme AK substrate is preferred.

Examples of protease assays are described in the experimental part.

There are no limitations on the origin of the protease for use according to the invention. Thus, the term ''protease'' includes not only natural or wild-type proteases, but also any mutants, variants, fragments etc. thereof, as well as synthetic proteases, such as shuffled proteases, and consensus proteases.

Examples of sub-groups of proteases are plant proteases, and microbial proteases.

Examples of proteases of plant origin are papain, ficin, bromelain, and the protease from the sarcocarp of melon fruit (Kaneda et al, J.Biochem. 78, 1287-1296 (1975).

In the present context, the term ''microbial'' mainly serves to exclude the endogenous (or natural, or wild-type) animal serine protease of trypsin.

In a particular embodiment, the term ''microbial'' indicates that the protease is derived from a microorganism, or is an analogue, a fragment, a variant, a mutant, or a synthetic protease derived from a microorganism. The term ''derived from'' means produced by a microorganism, be it a genetically modified, or a naturally occurring microorganism, i.e. the term covers the expression of wild-type, naturally occurring proteases, as well as expression of recombinant or synthetic proteases.

The term ''microorganism'' as used herein includes Archaea, bacteria, fungi, vira etc.

Examples of microorganisms are bacteria, such as bacteria of the genus Bacillus, e.g. Bacillus sp. PD498, NCIMB No.40484; or bacteria of the group of actinomycetes, such as Nocardiopsis sp. 10R, available from NRRL as NRRL 18262; or mutants or variants thereof.

In what follows, the proteases derived from the bacteria 10R and PD498 will also be referred to as 10R and PD498.

Further examples of microorganisms are fungi, such as yeast or filamentous fungi, e.g. chosen from the genera Paecilomyces, Aspergillus, Acremonium, Fusarium, Penicillium, Verticillium, or Microascus; or mutants or variants thereof.

The term ''animal'' includes all animals, including human beings. Examples of animals are mono-gastric animals, e.g. pigs (including, but not limited to, piglets, growing pigs, and sows); poultry such as turkeys and chicken (including but not limited to broiler chicks, layers); young calves; and fish (including but not limited to salmon).

The term ''feed'' or ''feed composition'' means any compound, preparation, mixture, or composition suitable for, or intended for intake by an animal. In one embodiment, the feed has a content of energy (e.g. therapeutics are excepted). In another embodiment, one or more vegetable proteins are included in the feed. These may derive partly from legumes, partly from cereals, e.g. from soybean, and from one or more of maize or wheat.

In the use according to the invention the protease can be fed to the animal before, after, or simultaneously with the diet. The latter is preferred.

In the present context, the term ''acid-stable'' means, that the protease activity of the pure protease enzyme, in a dilution corresponding to A_{280} - 1.0, as measured using an appropriate substrate, and following incubation for 2 hours at 37°C, in one or more of the following buffers:

a) 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 2.0;

b) 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 2.5;

c) 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 3.0; and/or

d) 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 3.5,

is at least 40% of the reference activity.

The term ''reference activity'' refers to the protease activity of the same protease, incubated in pure form, in a dilution corresponding to A_{280} = 1.0, with the same substrate, for 2 hours at 5°C, in the following buffer: 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 9.0.

In the above definition of acid-stability, a particular example of a (non-reference or sample) buffer pH-value is 2.0. Other particular examples are 2.5, 3.0, and 3.5.

In alternative embodiments, pH values of 6.0, 6.5, 7.0, 7.5, 8.0, or 8.5 can be applied for the reference activity incubation.

The term ''pure'' protease refers to a sample, which by a scan of a Coomassie stained SDS-PAGE gel is measured to have at least 95% of its scan intensity in the band corresponding to said protease (see Example 2).

The term ''A_{280} = 1.0'' means such concentration (dilution) of said pure protease which gives rise to an absorption of 1.0 at 280 nm in a 1cm path length cuvette relative to a buffer blank. The pure protease (in 1mM succinic acid, 2mM $CaCl_2$, 100mM

NaCl, pH 6.0 and with an A_{280} absorption > 10) is diluted in the assay buffer of the relevant pH value to A_{280} = 1.0.

The term "appropriate substrate" means a substrate that includes peptide bonds relevant for the specificity of the protease in question, for example one or more of the substrates Suc-AAPF-pNA, Boc-VLGR-pNA, or Suc-AAPE-pNA,

In particular embodiments of the above acid-stability definition, the protease activity is at least 45, 50, 55, 60, 65, 70, 75, 80, 85, or at least 90% of the reference activity.

In an alternative version of the above acid-stability definition, the (non-reference, or sample) protease activity is measured following incubation at one or more of the pH-values of 1.0, and 1.5.

In one particular embodiment, the serine proteases for use according to the invention, besides being acid-stable, also have a pH-activity optimum close to neutral.

The term "pH-activity optimum close to neutral" means one or more of the following: That the pH-optimum is in the interval of pH 6.0-11.0, or pH 6.0-10.5, or pH 6.0-10.0, or pH 6.5-10.5, or pH 7.0-10.0, or pH 7.5-10.0; or that the relative activity at pH 6.5 is at least 0.25; that the relative activity at pH 7.0 is at least 0.30, or at least 0.35, or at least 0.40, or at least 0.45, or at least 0.50, or at least 0.55; that the relative activity at pH 7.5 is at least 0.50; or that the relative activity at pH 8.0 is at least 0.60 - reference being made to Example 2, and Fig. 2 herein.

In another particular embodiment, the serine proteases for use according to the invention, besides being acid-stable, are also thermostable.

The term "thermostable" means one or more of the following: That the temperature optimum is at least 50°C, or at

least 52°C, or at least 55°C, or at least 57°C, or at least 60°C; or that the relative activity at 50°C is at least 0.15, or at least 0.20; that the relative activity at 55°C is at least 0.30; that the relative activity at 60°C is at least 0.40; that the relative activity at 65°C is at least 0.60; that the relative activity at 70°C is at least 0.35, or at least 0.40, or at least 0.50, or at least 0.60, or at least 0.70, or at least 0.75, or at least 0.80, or at least 0.85, or at least 0,90; or that the relative activity at 80°C is at least 0.20 - reference being made to Example 2 and Fig. 2 herein.

The term ''solubilisation of proteins'' basically means bringing protein(s) into solution. Such solubilisation may be due to a protease-mediated release of protein from other components of the usually complex natural compositions such as feed. ''Solubilisation'' can be measured as an increase in the amount of soluble proteins, by reference to a sample with no protease treatment (see Example 4 herein).

The term ''degradation of proteins'' with proteases generally refers to the enzymatic reaction in which the protein is degraded, catalyzed by the protease, into peptides and/or amino acids. ''Degradation'' can be measured as described in Example 4 herein.

The term ''solubilisation and/or degradation'' includes solubilisation, degradation, solubilisation and degradation, and solubilisation or degradation; as well as simply ''treating.''

The term ''vegetable proteins'' as used herein refers to any vegetable protein source (e.g. compound, composition, preparation or mixture) that includes protein. In particular embodiments, the protein content is at least 0.5, 1, 2, 4, 5, 6, 8 or 10% (w/w).

In the present context, examples of ''vegetable protein sources'' are plant materials derived from one or more of the families Fabaceae (Leguminosae), Cruciferaceae, Chenopodiaceae, and Poaceae. In a particular embodiment, the vegetable protein source is derived from one or more plants of the family Fabaceae, e.g. soybean, lupine, pea, or bean. In another particular embodiment, the vegetable protein source is derived from one or more plants of the family Chenopodiaceae, e.g. beet, sugar beet, spinach or quinoa. Other examples of vegetable protein sources are rapeseed, and cabbage. Soybean is a preferred protein source.

Other examples of vegetable protein sources are cereals such as barley, wheat, rye, oat, maize (corn), rice, and sorghum.

The treatment of the vegetable proteins with one or more acid-stable, serine protease can be performed in any way. The conditions at which the treatment takes place may reflect the characteristics of the protease in question. For example, the treatment may take place at a pH-value at which the relative activity of the actual protease is at least 50, or 60, or 70, or 80 or 90%. Likewise, for example, the treatment may take place at a temperature at which the relative activity of the actual protease is at least 50, or 60, or 70, or 80 or 90% (these relative activities being defined as in Example 2 herein).

In one embodiment the treatment is a pre-treatment of animal feed, i.e. the proteins are solubilised and/or degraded before intake.

In another embodiment, the treatment serves to produce peptides and/or amino acids for other uses, e.g. for use in food for humans, and/or for use as flavourants in various food or

The term ''improving the nutritional value of an animal feed'' means improving the availability of the proteins, thereby leading to increased protein extraction, higher protein yields, and/or improved protein utilization. The nutritional value of the feed is therefore increased, and the growth rate and/or feed conversion ratio (i.e. the weight of ingested feed relative to weight gain) of the animal is improved.

The protease can be added to the feed in any form, be it as a relatively pure protease (e.g. at least 60, 70, 80, 85, 90 or 95% pure), or in admixture with other components intended for addition to animal feed, i.e. in the form of animal feed additives, such as pre-mixes for animal feed.

Apart from the acid-stable, serine protease, the animal feed additives of the invention contain other ingredients, such as one or more ingredients chosen from the following non-exclusive, and non-limiting list of other feed-additive ingredients: Colouring agents, aroma compounds, stabilisers, vitamins, minerals, and/or other feed or food enhancing enzymes.

In particular embodiments of the animal feed use of the invention, and in the method of the invention for solubilisation and/or degradation of vegetable proteins, an additional addition or treatment with one or more enzymes is included, wherein these enzymes are selected from the group comprising other proteases, phytases, and lipolytic or glycosidase/carbohydrase enzymes. Examples of such enzymes are indicated in WO95/28850.

The protease should of course be applied in an effective amount, i.e. in an amount adequate for improving solubilisation and/or degradation, and/or improving nutritional value of feed. It is at present contemplated that the enzyme is administered in e.g. one or more of the following amounts: From about 0.1 mg to

about 10 mg enzyme/kg animal feed; or from about 0.1 mg to about 5 mg enzyme/kg animal feed.

Many vegetables contain anti-nutritional factors such as lectins and trypsin inhibitors. The most important anti-nutritional factors of soybean are the lectin soybean agglutinin (SBA), and trypsin inhibitors.

Lectins are proteins that bind to specific carbohydrate-containing molecules with considerable specificity, and when ingested they become bound to the intestinal epithelium. This may lead to reduced viability of the epithelial cells and reduced absorption of nutrients.

SBA is a glycosylated, tetrameric lectin with a subunit molecular weight of about 30 kDa and a high affinity for N-acetylgalactosamine.

Trypsin inhibitors affect the intestinal proteolysis reducing protein digestibility, and also increase the size of and secretion from the pancreas leading to a loss of amino acids in the form of digestive enzymes. Examples of trypsin inhibitors are the Bowman-Birk Inhibitor, that has a molecular weight of about 8 kDa, contains 7 disulfide bridges and has two inhibitory loops specific for trypsin-like and chymotrypsin-like proteases; and the Kunitz Inhibitor that contains one binding site for trypsin-like proteases and has a molecular weight of about 20 kDa.

The proteases for use according to the invention have been shown to hydrolyse anti-nutritional factors like SBA lectin, and the trypsin inhibitors Bowman Birk Inhibitor and Kunitz Factor. See the experimental part, Example 5.

Thus, the invention also relates to the use of acid-stable serine proteases for hydrolysing, or reducing the amount of, anti-nutritional factors, e.g. SBA lectin, and trypsin

inhibitors, such as the Bowman Birk Inhibitor, and the Kunitz Factor; as well as methods for hydrolysing and/or reducing the amount thereof.

Example 1.

Screening for acid-stable proteases

A large number of proteases were analysed for stability at pH 3, with the objective of identifying proteases that have the necessary stability to pass through the acidic stomach of mono-gastric animals.

The proteases had been purified by conventional chromatographic methods such as ion-exchange chromatography, hydrophobic interaction chromatography and size exclusion chromatography (see e.g. Protein Purification, Principles, High Resolution Methods, and Applications. Editors: Jan-Christer Janson, Lars Rydén. VCH Publishers, 1989).

Protease activity was determined as follows: The protease was incubated with casein at 25°C, pH 9.5 for 30 minutes, then TCA (tri-chloro acetic acid) was added to a final concentration of 2% (w/w), the mixture was filtrated to separate the sediment, and the filtrate was analysed for free primary amino acid groups (determined in a colometric assay based on OPA (o-phthal-dialdehyde) and measurement of absorbancy at 340nM, using a serine standard (Biochemische Taschenbuch teil II, Springer-Verlag (1964), p.93 and p.102). One Casein Protease Unit (CPU) is defined as the amount of enzyme liberating 1mmol of TCA-soluble primary amino acid groups per minute under standard conditions, i.e. 25°C and pH 9.5.

The proteases were diluted to an activity of 0.6 CPU/l in water, divided in two aliquots and each aliquot was then further diluted to 0.3 CPU/l with 100 mM citrate buffer, pH 3, and 100

mM phosphate buffer, pH 7 respectively. The diluted samples were incubated at 37°C for 1 hour, and 20 µl of the samples were applied to holes in 1% agarose plates containing 1% skim milk. The plates (pH 7.0) were incubated at 37°C over night and clearing zones were measured.

42 proteases performed well in this test. Two of these were selected first for further testing: PD498 and 10R.

The PD498 protease had been prepared as described in Example 1 of WO93/24623.

The 10R protease had been prepared as described in Examples I, II and V of WO 88/03947.

Both proteases belong to the group of serine proteases.

Example 2

Characterization and comparative study of pure serine proteases

The purpose of the characterization was to study the pH-stability, pH-activity and temperature-activity profiles of these two proteases 10R and PD498, in comparison to the known feed-related serine proteases of Sub.Novo, Sub.Novo(Y217L), and Savinase®.

Sub.Novo is subtilisin from Bacillus amyloliquefaciens, and Sub.Novo(Y217L) is the mutant thereof that is disclosed in WO96/05739. Sub.Novo was prepared and purified from a culture of the wild-type strain using conventional methods, whereas the mutant was prepared as described in Examples 1-2, and 15-16 of EP 130756.

Savinase® is a subtilisin commercially available from Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark.

Determination of purity of protease samples

The purity of the protease samples was determined by the following procedure:

40µl protease solution (A_{280} concentration = 0.025) was mixed with 10µl 50%(w/v) TCA (trichloroacetic acid) in an Eppendorf tube on ice. After half an hour on ice the tube was centrifuged (5 minutes, 0°C, 14.000 x g) and the supernatant was carefully removed. 20µl SDS-PAGE sample buffer (200µl Tris-Glycine SDS Sample Buffer (2x) (LC2676 from NOVEX™) + 160µl dist. water + 20µl ß-mercaptoethanol + 20µl 3M Tris-base) was added to the precipitate and the tube was boiled for 3 minutes. The tube was centrifuged shortly and 10µl sample was applied to a 4-20% gradient Tris-Glycine precast gel from NOVEX™ (EC60255). The electrophoresis was performed as described in 'NOVEX™, Precast Gel instructions'. After electrophoresis, the gel was rinsed 3 times, 5 minutes each, with 100 ml of distilled water by gentle shaking. The gel was then gently shaked with Gelcode® Blue Stain Reagent (PIERCE cat. No. 24592) for one hour and washed by gentle shaking for 8 to 16 hours with distilled water with several changes of distilled water. Finally, the gel was dried using the DryEase™ Gel Drying System from NOVEX™. Dried gels were scanned with a Arcus II scanner from AGFA equipped with Fotolook 95 v2.08 software and imported to the image evaluation software CREAM™ for Windows (Kem-En-Tec) by the *File/Acquire* command with the following settings (of Fotolook 95 v2.08): Original=Reflective, Mode=Color RGB, Scan resolution=240 ppi, Output resolution=120lpi, Scale factor=100%, Range=Histogram with Global selection and Min=0 and Max=215, ToneCurve=None, Sharpness=None, Descreen=None and Flavor=None, thereby producing an *.img picture file of the SDS-PAGE gel, which was used for

the menu command *Analysis/1-D*. Two scan lines were placed on the *.img picture file with the *Lane Place Tool*: A Sample scan line and a Background scan line. The Sample scan line was placed in the middle of a sample lane (with the protease in question) from just below the application slot to just above the position of the Bromphenol blue tracking dye. The Background scan line was placed parallel to the Sample scan line, but at a position in the pictured SDS-PAGE gel where no sample was applied, start and endpoints for the Background scan line were perpendicular to the start and endpoints of the Sample scan line. The Background scan line represents the true background of the gel. The width and shape of the scan lines were not adjusted. The intensity along the scan lines where now recorded with the *1-D/Scan* menu command with *Medium* sensitivity. Using the *1-D/Editor* menu command, the Background scan was subtracted from the Sample scan. Then the *1-D/Results* menu command was selected and the *Area* % of the protease peak, as calculated by the CREAM™ software, was used as the protease purity.

The following results were obtained:

Protease	Purity (Area %)
10R	100.0
PD498	96.3
Sub.Novo	95.5
Sub.Novo (Y217L)	96.0
Savinase®	99.2

Assays

pH-activity assay

pNA substrates: Suc-AAPF-pNA (Sigma® S-7388) was used for proteases: 10R, PD498, Sub.Novo, Sub.Novo(Y217L) and Savinase®.

Assay buffer: 100mM succinic acid (Merck 1.00682), 100mM HEPES (Sigma H-3375), 100mM CHES (Sigma C-2885), 100mM CABS (Sigma C-5580), 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, adjusted to pH-values 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, or 11.0 with HCl or NaOH.

Assay temperature: 25°C.

A 300µl protease sample (diluted in 0.01% Triton® X-100) was mixed with 1.5 ml of the assay buffer at the respective pH value, bringing the pH of the mixture to the pH of the assay buffer. The reaction was started by adding 1.5ml pNA substrate (50mg dissolved in 1.0ml DMSO and further diluted 45x with 0.01% Triton® X-100) and, after mixing, the increase in A_{405} was monitored by a spectrophotometer as a measurement of the protease activity at the pH in question. The assay was repeated with the assay buffer at the other pH values, and the activity measurements were plotted as relative activity against pH. The relative activities were normalized with the highest activity (pH-optimum), i.e. setting activity at pH-optimum to 1, or to 100%. The protease samples were diluted to ensure that all activity measurements fell within the linear part of the dose-response curve for the assay.

pH-stability assay

pNA substrates: Suc-AAPF-pNA (Sigma® S-7388) was used for proteases: 10R, PD498, Sub.Novo, Sub.Novo(Y217L) and Savinase®.

Assay buffer: 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100

adjusted to pH-values 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 or 11.0 with HCl or NaOH.

Each protease sample (in 1mM succinic acid, 2mM $CaCl_2$, 100mM NaCl, pH 6.0 and with an A_{280} absorption > 10) was diluted in the assay buffer at each pH value tested to A_{280} = 1.0. The diluted protease samples were incubated for 2 hours at 37°C. After incubation, protease samples were diluted in 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100, pH 9.0, bringing the pH of all samples to pH 9.0.

In the following activity measurement, the temperature was 25°C.

300µl diluted protease sample was mixed with 1.5ml of the pH 9.0 assay buffer and the activity reaction was started by adding 1.5ml pNA substrate (50mg dissolved in 1.0ml DMSO and further diluted 45x with 0.01% Triton® X-100) and, after mixing, the increase in A_{405} was monitored by a spectrophotometer as a measurement of the (residual) protease activity. The 37°C incubation was performed at the different pH-values and the activity measurements were plotted as residual activities against pH. The residual activities were normalized with the activity of a parallel incubation (control), where the protease was diluted to A_{280} = 1.0 in the assay buffer at pH 9.0 and incubated for 2 hours at 5°C before activity measurement as the other incubations. The protease samples were diluted prior to the activity measurement in order to ensure that all activity measurements fell within the linear part of the dose-response curve for the assay.

Temperature-activity assay

Protazyme AK tablets were used for obtaining temperature profiles. Protazyme AK tablets are azurine-crosslinked casein prepared as tablets by Megazyme.

Assay buffer: 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM $CaCl_2$, 150mM KCl, 0.01% Triton® X-100 adjusted to pH 9.0 with NaOH.

A Protazyme AK tablet was suspended in 2.0ml 0.01% Triton® X-100 by gentle stirring. 500µl of this suspension and 500µl assay buffer were mixed in an Eppendorf tube and placed on ice. 20µl protease sample (diluted in 0.01% Triton X-100) was added. The assay was initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which was set to the assay temperature. The tube was incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate. The assay incubation was stopped by transferring the tube back to the ice bath. The tube was centrifuged in an ice-cold centrifuge for a few minutes and the A_{650} of the supernatant was read by a spectrophotometer. A buffer blind was included in the assay (instead of enzyme). A_{650}(protease) - A_{650}(blind) was a measurement of protease activity. The assay was performed at different temperatures and the activity measurements were plotted as relative activities against incubation temperature. The relative activities were normalized with the highest activity (temperature optimum). The protease samples were diluted to ensure that all activity measurements fell within the near linear part of the dose-response curve for the assay.

An overview of the activity optima (pH- and temperature activity) is seen in Table 1. pH-stability, pH-activity and temperature-activity profiles are seen in figures 1-3, and a

detailed comparison of the pH-stability data for the proteases at acidic pH-values is seen in Table 2.

Table 1

pH- and temperature optima of various proteases

Protease	pH-optimum (pNA-substrate)	Temperature-optimum at pH 9.0 (Protazyme AK)
10R	10	70°C
PD498	9	60°C
Sub.Novo[1]	10	70°C
Sub.Novo(Y217L)[2]	9	70°C
Savinase®[3]	9	70°C

Table 2

pH-stability of various proteases, between pH 2.0 and 5.0

Protease	pH 2.0	pH 2.5	pH 3.0	pH 3.5	pH 4.0	pH 4.5	pH 5.0
10R	0.779	1.000	1.029	0.983	0.991	1.019	1.004
PD498	0.001	0.001	0.428	0.940	0.991	0.989	0.991
Sub.Novo	0.007	0.003	0.000	0.000	0.024	0.784	0.942
Sub.Novo(Y217L)	0.000	0.000	0.002	0.003	0.350	0.951	0.996
Savinase®	0.001	0.001	0.001	0.003	0.338	0.929	0.992

Example 3

Ability of proteases to degrade insoluble parts of Soy Bean Meal (SBM)

The two selected proteases, PD498 and 10R, were tested for their ability to make the insoluble/indigestible parts of SBM accessible to digestive enzymes and/or added exogeneous enzymes.

Their performance was compared to two known feed-related proteases, "Protease I" and "Protease II," prepared as described in WO 95/02044. "Protease I" is an Aspergillopepsin II type of protease, and "Protease II" and Aspergillopepsin I

aculeatus (reference being made to Handbook of Proteolytic Enzymes referred to above).

The test substrate, the so-called soy remnant, was produced in a process which mimics the digestive tract of monogastric animals, including a pepsin treatment at pH 2, and a pancreatin treatment at pH 7.

In the pancreatin treatment step a range of commercial enzymes was added in high dosages in order to degrade the SBM components that are accessible to existing commercial enzymes.

The following enzymes, all commercially available from Novo Nordisk A/S, Denmark, were added: Alcalase® 2.4L, Neutrase® 0.5L, Flavourzyme® 1000L, Energex L, BioFeed® Plus L, Phytase Novo L. The SBM used was a standard 48% protein SBM for feed, which had been pelletized.

After the treatment only 5% of the total protein was left in the resulting soy remnant.

FITC labelling protocol

The remnant was subsequently labelled with FITC (Molecular Probes, F-143) as follows: Soy remnant (25 g wet, ~ 5 g dry) was suspended in 100 ml 0.1M carbonate buffer, pH 9 and stirred 1 hour at 40°C. The suspension was cooled to room temperature and treated with fluorescein 5-isothiocyanate (FITC) over night in the dark. Non-coupled probe was removed by ultrafiltration (10.000 Mw cut-off).

FITC-assay

The FITC-labelled soy remnant was used for testing the ability of the proteases to degrade the soy remnant using the following assay: 0.4 ml protease sample (with A_{280} = 0.1) was mixed with 0.4 ml FITC-soy remnant (suspension of 10 mg/ml in 0.2M sodium-phosphate buffer pH 6.5) at 37°C, and the relative fluorescence units (RFU 485/535nm) measured after 0 hours and

after 22 hours incubation. A blind sample was prepared by adding 0.4 ml buffer instead of enzyme sample.

$RFU_{sample} = \Delta RFU_{sample} - \Delta RFU_{blind}$, where ΔRFU = RFU(22 hours) - RFU(0 hours)

The resulting "FITC values" (RFU_{sample} values) are shown in Table 3 below.

Table 3

Ability of proteases to degrade soy remnant

Protease	FITC
PD498	61900
10R	92900
Protease I	-9200
Protease II	-1200

Example 4

Test of proteases in an in vitro stomach and small intestine model

The two selected proteases PD498 and 10R, as well as Flavourzyme®, a protease-containing enzyme preparation from Aspergillus oryzae (commercially available from Novo Nordisk A/S, Denmark) were tested for their ability to solubilise and degrade maize-SBM proteins in an in vitro model.

Outline of in vitro model

Components added to flask	Time course (min)
10g maize-SBM (60:40) + HCl/pepsin (3000U/g diet) + protease (0.1mg enzyme protein/g diet or 3.3 mg Flavourzyme®/g diet), T=40°C, pH=3.0	t=0
NaOH, T=40°C, pH 6	t=60
$NaHCO_3$/pancreatin (8.0mg/g diet), T=40°C, pH 6-7	t=80
Stop incubation and take samples, T=0°C	t=320

Substrates

The SBM contains 43% protein and the maize 8.2% protein, which gives a total content of 2.21 g protein/flask.

Other components

Pepsin (Sigma P-7000; 539 U/mg solid (Anson J.Gen.Physiol. 22 (1938))

Pancreatin (Sigma P-7545; 8xU.S.P. (U.S. Pharmacopeia))

Enzyme protein determinations

The amount of enzyme protein is calculated on the basis of the A_{280} values and the amino acid sequences (amino acid compositions) using the principles outlined in S.C.Gill & P.H. von Hippel, Analytical Biochemistry 182, 319-326, (1989).

Experimental procedure for in vitro model

1. 10 g of substrate is weighed into a 100 ml flask.

2. At time 0 min, 46 ml HCl (0.1M) containing pepsin (3000U/g diet) and 1 ml of protease (0.1mg enzyme protein/g diet for 10R or PD498, or 3.3 mg Flavourzyme®/g diet) is added to the flask while mixing. The flask is incubated at 40°C.

3. At time 20-25 min, pH is measured.

4. At time 45 min, 16 ml of H_2O is added.

5. At time 60 min, 7 ml of NaOH (0.4M) is added.

6. At time 80 min, 5 ml of $NaHCO_3$ (1M) containing pancreatin (8.0 mg/g diet) is added.

7. At time 90 min, pH is measured.

8. At time 300 min, pH is measured.

9. At time 320 min, 2 samples of 30 ml are taken to an ice-bath before centrifugation (15000 x g, 10 min, 0°C). Supernatant is stored as 2 aliquots.

10. One aliquot is used for determination of total soluble protein, the other aliquot is subjected to gel-filtration for separation and determination of low-molecular weight (LMW) proteins (using PD10 columns from Pharmacia containing Sephadex® G25).

Gel filtration

2.5 ml of supernatant is added to the PD10 column equilibrated in buffer (0.1M phosphate buffer, pH 6.5), and the LMW-fraction is eluted in 6.5 ml buffer (0.1 M phosphate buffer, pH 6.5) after the HMW-fraction has been eluted with 3.5 ml buffer.

Protein determination

The LMW eluate and the supernatant are thawed and analysed for protein content using the Kjeldahl method (determination of % nitrogen; A.O.A.C. (1984) Official Methods of Analysis 14th ed. Association of Official Analytical Chemists, Washington DC).

Calculations

For all samples, in vitro protein solubility and degradation digestibility are calculated using the equations below.

Amount of protein in diet sample: 22.1% of 10 g = 2.21 g

If all the protein were solubilised in the 75 ml of liquid, the protein concentration in the supernatant would be: 2.21 g/75 ml ≈ 2.95%

Note that the supernatants also include the digestive and exogenous enzymes. In order to determine the solubility, the protein contribution from the digestive and exogenous enzymes should be subtracted from the protein concentrations in the supernatants whenever possible.

% protein from the pancreatin (X mg/g diet) and pepsin (Y U/g diet) =
((Xmg pancreatin/g diet x 10g diet x 0.69 x 100%)/(1000mg/g x 75g)) + ((YU pepsin/g diet x 10g diet x 0.57 x 100%)/(539U/mg x 1000mg/g x 75g))

where 0.69 and 0.57 refer to the protein contents in the pancreatin and pepsin preparations used (i.e. 69% of the pancreatin, and 57% of the pepsin is protein as determined by the Kjeldahl method referred to above).

% protein from exogenous enzymes (Z mg EP/g diet)=
(Zmg EP/g diet x 10g diet x 100%)/ (1000mg/g x 75g)

% protein corrected in supernatant = % protein in supernatant as analyzed - (% protein from digestive enzymes + % protein from exogenous enzymes)

Protein solubilisation (%) =
(% protein corrected in supernatant x 100%)/2.95 %

If all the protein from the diet is solubilised and degraded to low molecular weight proteins, the concentration in the eluted low molecular fraction would be:

(2.95% x 2.5ml supernatant for gel filtration)/(6.5ml LMW-eluent) = 1.13%

Protein digestibility (degradation) (%) = (%protein in LMW-eluent x 100%)/1.13%

The results are shown in Table 4 below.

Table 4

Enzyme	Solubilised P (% of total)	Degraded P(% of total)
Blind (no exogenous enzymes)	73.8[c]	46.6[c]
+10R	77.5[a]	49.7[a]
+PD498	75.6[b]	48.2[a, b]
+Flavourzyme®	74.1[c]	47.4[b, c]

[a,b,c] Values within a column not sharing a common superscript letter are significantly different, $P<0.05$.

It can be seen from the results in Table 4 that 10R and PD498 (dosed at 1 mg enzyme protein/10 g diet) significantly increased solubilisation and degradation of maize-SBM proteins compared to the control samples containing digestive enzymes only (pepsin (32 mg protein/10 g diet) and pancreatin (55 mg protein/10 g diet)).

Example 5

Degradation of the lectin SBA and the soybean Bowman-Birk and Kunitz Inhibitors

The ability of the proteases to hydrolyse soybean agglutinin (SBA) and the soy Bowman-Birk and Kunitz trypsin inhibitors was tested.

Pure SBA (Fluka 61763), Bowman-Birk Inhibitor (Sigma T-9777) or Kunitz Inhibitor (Trypsin Inhibitor from soybean, Boehringer Mannheim 109886) was incubated for 2 hours, 37°C, pH 6.5 with the proteases PD 498 and 10R (protease: anti-nutritional factor = 1:10, based on A_{280}). Incubation buffer: 50 mM dimethyl glutaric acid, 150 mM NaCl, 1 mM $CaCl_2$, 0.01% Triton X-100, pH 6.5.

The ability of the proteases to degrade SBA and the protease inhibitors was estimated from the disappearance of the native SBA or trypsin inhibitor bands and appearance of [illegible]

molecular weight degradation products on SDS-PAGE gels. Gels were stained with Coomassie blue and intensities of bands determined by scanning.

The results, as % of anti-nutritional factor degraded, are shown in Table 5 below.

It is contemplated that the ability to degrade the anti-nutritional factors in soy can also be estimated by applying the Western technique with antibodies against SBA, Bowman-Birk Inhibitor or Kunitz Inhibitor after incubation of soybean meal with the candidate proteases (see WO98/56260).

Table 5

	SBA	Bowman-Birk Inhibitor	Kunitz Inhibitor
PD 498	21	41	100
10R	75	25	100

CLAIMS

1. Use of one or more acid-stable, serine proteases in animal feed.

2. A method for the solubilisation and/or degradation of vegetable proteins, wherein the vegetable proteins are treated with one or more acid-stable, serine proteases.

3. A method for improving the nutritional value of an animal feed, wherein one or more acid-stable, serine proteases are added to the feed.

4. An animal feed additive comprising one or more acid-stable, serine proteases.

5. An animal feed composition comprising one or more acid-stable, serine proteases.

ABSTRACT

Use of one or more acid-stable, serine proteases in animal feed, and methods for the solubilisation and/or degradation of vegetable proteins using such proteases.

Fig. 1

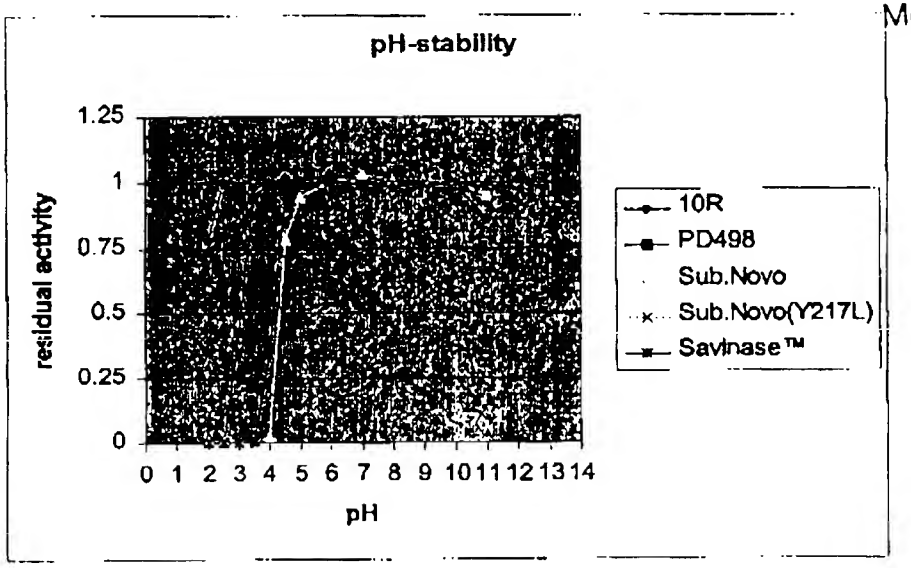

Fig. 2

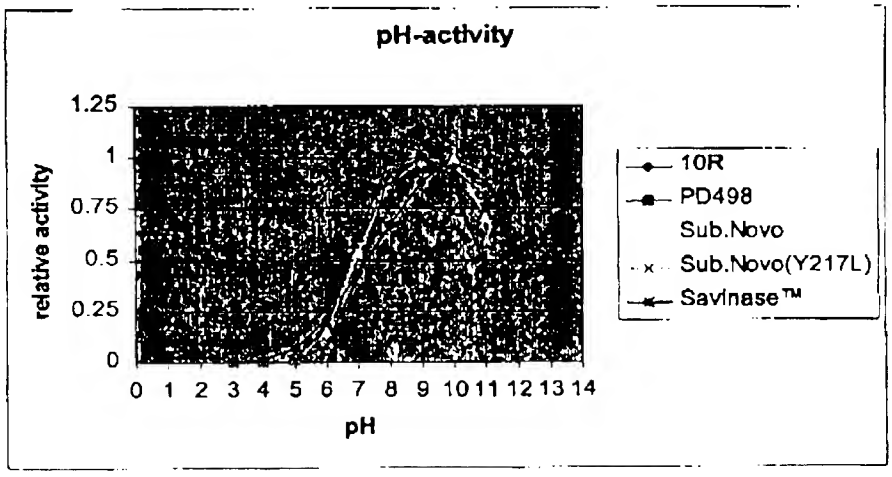

Fig. 3